Citation for published version (APA):
RESEARCH PAPER

Divergent modulation of Rho-kinase and Ca\textsuperscript{2+} influx pathways by Src family kinases and focal adhesion kinase in airway smooth muscle

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BACKGROUND AND PURPOSE
The importance of tyrosine kinases in airway smooth muscle (ASM) contraction is not fully understood. The aim of this study was to investigate the role of Src-family kinases (SrcFK) and focal adhesion kinase (FAK) in GPCR-mediated ASM contraction and associated signalling events.

EXPERIMENTAL APPROACH
Contraction was recorded in intact or α-toxin permeabilized rat bronchioles. Phosphorylation of SrcFK, FAK, myosin light-chain-20 (MLC\textsubscript{20}) and myosin phosphatase targeting subunit-1 (MYPT-1) was evaluated in cultured human ASM cells (hASMC). [Ca\textsuperscript{2+}] was evaluated in Fura-2 loaded hASMC. Responses to carbachol (CCh) and bradykinin (BK) and the contribution of SrcFK and FAK to these responses were determined.

KEY RESULTS
Contractile responses in intact bronchioles were inhibited by antagonists of SrcFK, FAK and Rho-kinase, while after α-toxin permeabilization, they were sensitive to inhibition of SrcFK and Rho-kinase, but not FAK. CCh and BK increased phosphorylation of MYPT-1 and MLC\textsubscript{20} and auto-phosphorylation of SrcFK and FAK. MYPT-1 phosphorylation was sensitive to inhibition of Rho-kinase and SrcFK, but not FAK. Contraction induced by Sr Ca\textsuperscript{2+} depletion and equivalent [Ca\textsuperscript{2+}] responses in hASMC were sensitive to inhibition of both SrcFK and FAK, while depolarization-induced contraction was sensitive to FAK inhibition only. SrcFK auto-phosphorylation was partially FAK-dependent, while FAK auto-phosphorylation was SrcFK-independent.

CONCLUSIONS AND IMPLICATIONS
SrcFK mediates Ca\textsuperscript{2+}-sensitization in ASM, while SrcFK and FAK together and individually influence multiple Ca\textsuperscript{2+} influx pathways. Tyrosine phosphorylation is therefore a key upstream signalling event in ASM contraction and may be a viable target for modulating ASM tone in respiratory disease.

Abbreviations
ASM, airway smooth muscle; hASMC, cultured human airway smooth muscle cells; KPSS, PSS with 80 mM equimolar substitution of Na\textsuperscript{+} for K\textsuperscript{+}; MLC\textsubscript{20}, myosin light-chain 20 KDa subunit; MLCP, myosin light-chain phosphatase; MYPT-1, myosin phosphatase targeting subunit-1; ROCE, receptor-operated Ca\textsuperscript{2+} entry; SOCE, store-operated Ca\textsuperscript{2+} entry; VOCE, voltage-operated Ca\textsuperscript{2+} entry
Introduction

Airway smooth muscle (ASM) tone is subject to regulation by cholinergic, catecholamine and NANC neurotransmitters as well as local inflammatory mediators. In healthy airways, muscle tone is normally low, providing a low resistance path for airflow. However, contraction may be enhanced in response to chemical irritants or allergens, particularly in lower respiratory tract bronchioles (Gilbert and Auchincloss, 1989; Pinelli et al., 2009). In asthma, airway resistance is increased, partly due to increased basal tone and hypersensitivity to constrictor stimuli in these bronchioles (Doeing and Solway, 2013; Meurs et al., 2008).

Smooth muscle contractile force depends on the degree of myosin light-chain-20 (MLC20) phosphorylation, which is in turn determined by the balance between Ca\textsuperscript{2+}-dependent activation of myosin light-chain kinase (MLCK) and Ca\textsuperscript{2+}-independent inhibition of myosin light-chain phosphatase (MLCP), as well as the formation and recruitment of myofilaments (Gunst et al., 2003; Somlyo and Somlyo, 2003). Increases in [Ca\textsuperscript{2+}]\textsubscript{i} result from Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) and a combination of Ca\textsuperscript{2+} entry through receptor-operated, store-operated and voltage-operated Ca\textsuperscript{2+} channels (ROCE, SOCE and VOCE, respectively). Inhibition of MLCP occurs via phosphorylation of myosin phosphatase targeting subunit-1 (MYPT-1), primarily by Rho-kinase (Feng et al., 1999), resulting in a further increase in MLC20 phosphorylation and contraction without the need for a further increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Somlyo and Somlyo, 2003). Although it is likely that bronchoconstrictors act via a combination of the above pathways, the precise mechanisms through which Ca\textsuperscript{2+} influx and Rho-kinase activity are mediated by GPCRs are not fully understood.

Src family kinases (SrcFK) and focal adhesion kinase (F AK) are widely expressed non-receptor tyrosine kinases (TKs) important in many aspects of cellular function, being activated in response to various stimuli including growth factors, GPCRs, reactive oxygen species and adhesion. SrcFK and F AK are often described as being mutually dependent or reciprocally activated, especially when associated with integrin engagement and/or growth factor receptor activation (Owen et al., 1999; Ishigaki et al., 2011). An effect of TKs on ASM tone was first suggested by the relaxant effect of non-selective tyrosine kinase inhibitors on rat isolated bronchioles (Chopra et al., 1997). Subsequently, selective inhibition of SrcFK and F AK was shown to depress GPCR-induced contraction in human, rodent or canine upper airways (Tang and Gunst, 2001; Katsumoto et al., 2013). F AK was linked to elevated [Ca\textsuperscript{2+}]\textsubscript{i} in response to various stimuli in trachea, but the relative influence of the kinase on VOCE, ROCE or SOCE or on Rho-kinase was not determined (Tang et al., 1999; Tang and Gunst, 2001). SrcFKs have been identified as upstream mediators of Rho-kinase in vascular smooth muscle (Nakao et al., 2002; Knock et al., 2008), but neither this relationship nor the influence of SrcFK on GPCR [Ca\textsuperscript{2+}]\textsubscript{i} responses has yet been examined in ASM. To our knowledge, only one previous study has examined the involvement of SrcFK or F AK specifically in the contraction of intralobar bronchioles, and this was limited to the role of SrcFK in mediating sensitization of rat bronchioles to mucaric agonists (Sakai et al., 2010).

In this study, we hypothesized that SrcFK and F AK mediate GPCR-induced ASM contraction via multiple signalling pathways and examined their influence on Rho-kinase-dependent MLCP inhibition/Ca\textsuperscript{2+}-sensitization and on SOCE/ROCE and VOCE Ca\textsuperscript{2+} entry pathways, in intra-lobar bronchioles of rat and cultured human ASM cells (hASM). We found that SrcFK, most likely c-Src itself, modulate Rho-kinase dependent Ca\textsuperscript{2+}-sensitization, but F AK does not, and that the two tyrosine kinases differentially regulate SOCE/ROCE and VOCE. We also suggest the existence of two subpopulations of GPCR-activated SrcFK, one being F AK-dependent and the other F AK-independent.

Methods

**Rats and tension measurement by wire myography**
All animal care and experimental procedures complied with UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039) and were...
deemed as humane as possible. All results involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010). A total of 98 rats were used. Male Wistar rats (~250 g) had free access to food and water and were maintained on a 12:12 h light/dark schedule. The rats were killed by an i.p. injection of sodium pentobarbital and the lungs and trachea were immediately removed. First or second-order intralobar bronchioles (~2 mm length) were dissected free of surrounding parenchyma and mounted on a wire myograph (DMT, dk), bathed in PSS (in mM: 118 NaCl; 24 NaHCO₃; 1 MgSO₄; 4 KCl; 5.56 glucose; 0.435 NaH₂PO₄; 1.8 CaCl₂, pH 7.4), gassed with 95% air, 5% CO₂ at 37°C. Bronchioles were incrementally stretched and alternately exposed to PSS containing 80 mM [K⁺] (equimolar substitution for Na⁺, K-PSS) until the point on the length tension curve at which muscle length was optimum for active tension development was achieved, as described previously (Moir et al., 2003). Viability for contraction experiments was confirmed by a response of at least 3 mN to the last challenge with KPSS. Bronchiole internal diameter after stretch was typically in the range 300–800 μm.

Specific examination of the Rhö-kinase dependent Ca²⁺-sensitization component of contraction was achieved by permeabilizing myograph-mounted bronchioles with α-haemolysin (α-toxin). PSS was first exchanged for relaxing solution (pCa = 10, in mM: 200 PIPES; 100 Mg(Mg)₂; 1000 KCl; 100 CaEGTA; 5 Na₂ATP; 10 Na₂creatine phosphate, pH 7.1), gassed with air at 26°C. α-toxin (60 μg ml⁻¹) was then applied in relaxing solution with pCa raised to 6.7 for 30 min, permeabilization being confirmed by the development of active tension. pCa was adjusted via proportionate substitution of K₂EGTA for CaEGTA, with 100 CaEGTA, 0 K₂EGTA being equivalent to pCa4.5. Contractile responses to bronchoconstrictors were conducted at pCa 6.5 (~300 nM [Ca²⁺]), which induced a contraction equivalent to 10-20% of that achieved by pCa 4.5. GTP 1 μM and 10 μM cyclopiazonic acid (CPIB) were included to support G-protein signalling and to prevent the influence of SR Ca²⁺ release on contraction respectively.

**Human tissue and cell culture**

Donations of human tissue were obtained following written informed consent and with the approval of the South East London Research Ethics Committee, REC reference number 10/H0804/66. All clinical procedures conformed to the standards set by the latest Declaration of Helsinki. hASMCs were obtained from healthy volunteers (n = 11; 7 women, 4 men; age range 22–53 years; life-long absence of respiratory symptoms; lung functions within normal limits) by deep endobronchial biopsy. ASM bundles were bathed in DMEM containing 10% FBS, L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids and amphotericin B (2 μg ml⁻¹), and subjected to enzymatic digestion in nominally Ca²⁺-free HEPES buffer containing: 5.56 mM glucose, 2 mg ml⁻¹ collagenase Type XI, 1 mg ml⁻¹ papain, 1 mg ml⁻¹ trypsin inhibitor and 1 mM DTT, for 30 min at 37°C. Cells were then dispersed into culture flasks containing DMEM (plus supplements) and incubated at 37°C, pH 7.4. Smooth muscle phenotype was confirmed by positive staining with anti-smooth muscle α-actin, anti-desmin and anti-calponin, with Alexa Fluor®488 labelled secondary antibody (Lifetechologies) and with TRITC-labelled phalloidin to confirm the presence of stress fibres in resting cells (Supporting Information Fig. S1). Cells were used for experiments at passages 4–9, grown to confluence and serum starved for 7 days in DMEM plus supplements, and the addition of 1% BSA, 5 μg ml⁻¹ transferrin, 1 μM insulin and 100 μM ascorbate.

**siRNA design and transfection**

Two siRNAs against human SRC (GenBank accession no. NM_005417) were designed as described previously (Reynolds et al., 2004; Ui-Tei et al., 2004). The 19 nucleotide target sequences (SRC-siRNA1: position 1489–1507 and SRC-siRNA2: position 1684–1702) were synthesized into 46–65 mer oligonucleotides with BamHI/HindIII overhangs (Sigma Aldrich) and cloned into the expression vector pSilencer 3.0-H1, containing pmxGFP (Ambion Inc.). All clones were purified using an EndoFree Plasmid Maxi Kit (Qiagen Ltd) and sequenced (Geneservice Ltd). hASMCs were transfected using the Basic Nucleofector® Kit and nucleofector device (Amaxa Biosystems). After 72 h, the transfection efficiency was >90%, confirmed by fluorescence microscopy.

**Protein lysate preparation and western blot**

Cultured hASMCs were treated in serum-free DMEM at 37°C. Preliminary studies showed that phosphorylation responses, although sustained for at least 5 min, peaked at ~30 s, so all subsequent acute treatments were for 30 s. Cells were immediately washed twice with ice-cold PBS, followed immediately by application of cell lysis buffer (NEB) containing 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCL, pH 7.5, for 1 h using an Xcell SureLock Mini-Cell (Invitrogen) and sonicated (20 s). Protein lysates were centrifuged at 9.2x10⁴ rpm for 10 min at 4°C, followed by a final wash in TBS-T. Lysates were then probed with primary antibodies (typically 1:1000 dilution) in TBS-T, 5% skimmed milk and 0.1% Tween-20, overnight at 4°C. Following washes in TBS-T, HRP-conjugated secondary antibody (typically 1:5000 dilution) was applied for 1 h at room temperature, followed by a final wash in TBS-T. ‘Phospho’ proteins were visualized with Super-Signal West


5267
Femto chemi-luminescent Substrate (Thermo scientific). Membranes were then stripped in Restore western blot stripping buffer (Thermo Scientific), re-blocked and re-incubated with corresponding ‘total’ antibody and appropriate secondary antibodies, as above. ‘Total’ proteins were visualized with either ECL plus or ECL prime (Amersham, GE healthcare). Images were captured and quantified using the ChemiDoc XRS+ gel-imaging system (Biorad). An estimate of the proportion of target protein that was phosphorylated was calculated as a ratio of ‘phospho’ over ‘total’ signal for each protein band from each gel, and the effects of acute treatments on these ratios was expressed as a percentage of control (untreated samples run on the same gel).

\[ Ca^{2+} \text{] measurement} \]

Cultured hASMCs were grown on glass cover-slips until 70% confluent, followed by 7 days of serum starvation. Cells were loaded with 1 μM Fura PE-3/AM in HBSS (containing in mM: 0.49 MgCl2, 0.41 MgSO4, 4 KCl, 0.44 KH2PO4, 4.2 NaHCO3, 120 NaCl, 0.34 Na2HPO4, 20 HEPES and 2 CaCl) at room temperature for 40 min. Coverslips were mounted on an upright microscope and cells perfused with HBSS, containing test reagents as required. Changes in \[ Ca^{2+} \text{] were measured as a ratio of 340 nm over 380 nm emission intensities with a ×20 oil immersion UV objective and a microspectrofluorimeter (CaïrnResearch Ltd., U.K.). For each coverslip, ratios obtained in zero \[ Ca^{2+} \text{] and the absence of drug were taken as background fluorescence (auto-fluorescence + residual basal \[ Ca^{2+} \text{]) and subtracted from all subsequent measurements.

Materials and reagents

Antibodies were obtained from cell signalling (anti-phospho-Src (tyr416); anti-Src; anti-phospho-FAK (Y397); anti-phospho-FAK (Y576/577); anti-FAK; anti-phospho-MLC (S19); anti-MYPT1; anti-MLC), Millipore (anti-phospho-MYPT1 (T696)), Sigma (anti-rabbit IgG; anti-mouse IgG). Kinase inhibitors were obtained from Sigma ((1R, 4r)-4((R)-1-aminoethyl)-3,4-dihydro-1H-quinolin-2-one (PF-573228); 6-[4-(3-methanesulfonylbenzylamino)-5-trifluoromethyl-5-pyrimidin-2-ylamino]-3,4-dihydro-1H-quinolin-2-one (PF-573228); N-[2-[[2-[2,3-dihydro-2-oxo-1H-indol-5-yl]amino]-5-(trifluoromethyl)-4-pyrimidinyl]amino[methyl]phenyl]-N-methyl-methanesulfonamide hydrate (PF-431396) or Calbiochem: 4-amino-5-(4-chlorophenyl)-7-(dimethylthethyl) pyrazolo[3,4-d]pyrimidine (PP2); 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3). Cell culture and western blot materials were obtained from Cell Signalling, Invitrogen, GE Healthcare or Thermo Scientific. Nifedipine, YMS8483, and cyclopiazonic acid and α-haemolysin were from Sigma.

Data analysis and statistics

All values are expressed as mean ± SEM. Non-linear regression curve fitting was performed with SigmaPlot 10. Carbachol (CCh) concentration-response curves were fitted using the Hill equation for the calculation of PD2 (-LogM EC50) and maximum response (Max). Bradykinin concentration responses were biphasic, and were best fitted using a two-site saturation model, for the characterization of a high affinity component (PD2-1 and Max-1) and a low affinity component (PD2-2 and Max-2). Statistical analysis of data was by Student’s paired or un-paired t-test (two groups of data, single factor), one-way ANOVA (more than two groups of data, single factor) or two-way ANOVA (more than two groups of data, two factors), with Holm–Sidak post tests where appropriate, and as indicated in figure or table legends, using SigmaPlot 10. Differences were considered significant if \( P < 0.05 \).

Results

GPCR-mediated contraction of rat bronchioles is dependent on SrcFK, Rho-kinase and FAK. We examined the contractile responses to CCh and bradykinin (BK) in rat bronchioles, whereby the bronchoconstrictors were applied cumulatively at 5 min intervals. Two concentration-response curves were performed in each bronchiolar (0.01–100μM), the first acting as a control and the second after pre-incubation with either the SrcFK inhibitor PP2 (30 μM), the Rho-kinase inhibitor Y27632 (10 μM), the FAK inhibitor PF-573228 (10 μM) or no inhibitor (control). In addition, to account for possible off-target effects of PP2 and PF-573228, key contractile responses were also repeated with PP3 (30 μM), the negative control for PP2 and a dual FAK/PYK2 inhibitor, PF-431396 (10 μM). CCh caused a sustained contraction at each dose (Figure 1A). The maximum response to CCh was significantly reduced by PP2 (\( P < 0.01 \), paired t-test, \( n = 8 \)), Y27632 (\( P < 0.01 \), paired t-test, \( n = 6 \)) and PF-573228 (\( P < 0.05 \), paired t-test, \( n = 8 \)), and the PD2 was significantly increased by PP2 (\( \sim 5.55 ± 0.09 \) vs. control –5.8 ± 0.14, \( P < 0.05 \), paired t-test, \( n = 8 \)), Y27632 (\( \sim 5.4 ± 0.07 \) vs. control –5.82 ± 0.07, \( P < 0.01 \), paired t-test, \( n = 6 \)) and PF-573228 (\( \sim 5.21 ± 0.08 \) vs. control –5.69 ± 0.07, \( P < 0.01 \), paired t-test, \( n = 8 \)) (Figure 1A-D). PP3 had no significant effect on either PD2 (\( \sim 5.6 ± 0.05 \) vs. control –5.72 ± 0.08, \( n = 7 \)) or maximum contraction (144 ± 5% vs. control 149 ± 3%, \( n = 7 \)). Conversely, PF-431396 had similar effects as those of PF-573228, causing a similar increase in PD2 (\( ~ 5.20 ± 0.05 \) vs. control –5.90 ± 0.07, \( P < 0.001 \), paired t-test, \( n = 7 \)) and a similar reduction in maximum contraction (139 ± 6.3% vs. control 176 ± 9.3%, \( P < 0.001 \), paired t-test, \( n = 7 \)) (Supporting Information Figs. S2A and S3A). In time-matched control responses, repeated in the absence of inhibitor, the maximum contraction of the second response was slightly increased (first repeat: 203 ± 22% vs. second repeat 228 ± 25%, \( P < 0.01 \), paired t-test, \( n=10 \)), but there was no significant change in PD2 (first repeat: –5.67 ± 0.11 vs. second repeat –5.63 ± 0.09, \( n = 10 \)).

Bradykinin caused a prominent transient contraction and a smaller sustained component at each dose (Figure 1E). The concentration-dependence of these responses appeared biphasic. Contraction at all concentrations of BK was biphasic, and were best fitted using a two-site saturation model, for the characterization of a high affinity component (PD2-1 and Max-1) and a low affinity component (PD2-2 and Max-2). Statistical analysis of data was by Student’s paired or un-paired t-test (two groups of data, single factor), one-way ANOVA (more than two groups of data, single factor) or two-way ANOVA (more than two groups of data, two factors), with Holm–Sidak post tests where appropriate, and as indicated in figure or table legends, using SigmaPlot 10. Differences were considered significant if \( P < 0.05 \).
for all BK dose responses curve fit data). BK-induced responses were nearly abolished by Y27632 (Figure 1G) and abolished by PF-573228 (Figure 1H), rendering curve fitting impossible. In time-matched control responses, repeated in the absence of inhibitor, there were no changes in either Max or PD₂ values for either the high or the low affinity component (not shown).

**SrcFKs mediate GPCR-induced Ca²⁺-sensitization and Rho-kinase activation, but FAK does not.** To clarify whether the effects of...
kinase inhibitors on bronchoconstrictor-induced contraction is mediated through a Rho-kinase-dependent Ca\textsuperscript{2+} sensitization pathway. CCh or BK concentration-response curves were repeated in \(\alpha\)-toxin-permeabilized rat bronchioles, with \([\text{Ca}^{2+}]_{i}\) fixed at pCa 6.5, in the absence or presence of PP2, Y27632 or PF-573228. After \(\alpha\)-toxin-permeabilization, bronchoconstrictor concentration-response curves were not repeatable (not shown), so controls and effects of antagonists were compared in separate bronchioles. CCh-induced contraction was almost absent in the presence of Y27632 and was significantly smaller in the presence of PP2 (\(P<0.05\), unpaired t-test, \(n=9\)), but in the presence of PF-573228 was not different from controls (Figure 2B). The \(P_{D_{2}}\) was significantly greater after PP2 (\(-4.64 \pm 0.13\), vs. control \(-5.08 \pm 0.12\), \(P<0.05\), unpaired t-test, \(n=9\)), but was no different in PF-573228. The underlying pCa 6.5 contraction was unaffected by either PP2 or PF-573228, but was partially inhibited by Y27632 (61 \(\pm\) 8% block, \(P<0.05\) vs. absence of Y27632, paired t-test, \(n=6\)). BK also produced a modest (relative to CCh) concentration-dependent contractile response in permeabilized bronchioles (Figure 2A and C). The concentration-dependence of these responses again appeared biphasic, but their small amplitude and poor sustainability rendered curve-fitting impossible. Nevertheless, peak responses were significantly smaller or absent in the presence of PP2 or Y27632, respectively, and no different in PF-573228 (Figure 2C).

To confirm that Rho-kinase is being activated by 30 s exposure to BK (1 \(\mu\)M) or CCh (100 \(\mu\)M), and whether this activation relates to subsequent activation of MLCK, we measured phosphorylation of MYPT-1 at T696, a known activation relates to subsequent activation of MLCK, we also examined the effects of PP2 and PF-753228 on SOCE/ROCE and VOCE. We found that the initial SR-emptying stimulus. After the recording of an initial baseline in 2 mM Ca\textsuperscript{2+}, and then for 5 min in nominally Ca\textsuperscript{2+}-free buffer, the addition of BK (1 \(\mu\)M) caused a near instantaneous increase in \([\text{Ca}^{2+}]_{i}\). This decayed back to the baseline within \(-2\) min; a response validating the choice of kinase inhibitor concentrations used. In rat trachea muscle, SrcFK and FAK auto-phosphorylation were also enhanced by BK and CCh, as was phosphorylation of MLC\(_{20}\) (S19) and MYPT-1 (Y397), (Figure 3E, F). Bronchoconstrictor-induced FAK Y397 phosphorylation was noticeably weaker in rat trachealis than in hASMC.

**FAK and SrcFK influence SOCE/ROCE and VOCE.** We examined the effects of PP2 and PF-753228 on VOCE-mediating contraction by sub-maximal depolarization with 40 mM KPSS. In control experiments, contraction amplitude induced by two consecutive KPSS exposures was not significantly different, when PP2 (30 \(\mu\)M) or PF-573228 (10 \(\mu\)M) was applied between the first and second exposures, the contractile response was modestly but significantly reduced by PF-573228, but not by PP2 (Figure 4A and B). To rule out a direct Ca\textsuperscript{2+}-channel antagonist effect of PF-573228, we also examined its effect on contraction induced by maximal depolarization with 80 mM KPSS. This contraction was significantly less sensitive to PF-573228 than the 40 mM KPSS contraction (11.5 \(\pm\) 2.7% inhibition, \(n=6\), vs. 30.2 \(\pm\) 6.3% inhibition of 40 mM KPSS, \(n=7\); \(P<0.05\) unpaired t-test). We then examined the effects of PP2 and PF-573228 on SOCE-mediated contraction. SR Ca\textsuperscript{2+} was first depleted with cyclopiazonic acid (CPA, 10 \(\mu\)M) in the absence of extracellular Ca\textsuperscript{2+} and presence of 200 \(\mu\)M EGTA, then 2 mM Ca\textsuperscript{2+} was re-applied. CPA was used here instead of a GPCR agonist because it would have been difficult to separate effects of the agonist on Ca\textsuperscript{2+} entry from those on Rho-kinase-mediated Ca\textsuperscript{2+} sensitization. In control experiments, re-application of 2 mM Ca\textsuperscript{2+} induced a biphasic contraction, which peaked at \(-2\) min and slowly decayed to \(-20\%\) of 80 mM KPSS after 30 min. In the presence of either PP2 or PF-573228, the sustained component was significantly smaller, decaying to \(-10\%\) of 80 mM KPSS after 30 min (Figure 4C and D). PP3 was without significant effect on this response, ruling out the possibility of a non-specific SOCE blocking effect of PP2 (Supporting Information Fig. S2B), while PF-431396 inhibited the response in a near-identical way to that of PF-573228, supporting a specific role for FAK in this response (Supporting Information Fig. S3B). To see whether these effects of PP2 and PF-573228 were due to an effect of SrcFK or FAK on SOCE itself or on secondary activation of VOCE, the effect of the SOCE blocker YMS8483 (10 \(\mu\)M) and the Ca\textsuperscript{2+} channel antagonist nifedipine (2 \(\mu\)M) on the SOCE-mediated contraction was also determined. Apart from a small residual transient contraction, the response was abolished by YMS8483, while nifedipine was without effect, apart from a small reduction in the peak response (Figure 4D).

To support contraction data and to further eliminate the possibility that SrcFK and FAK were indirectly influencing SOCE via an action on SR Ca\textsuperscript{2+} release, we also examine the effects of PP2 and PF-753228 on SOCE \([\text{Ca}^{2+}]_{i}\) responses in Fura-2 loaded hASMC, using BK as the initial SR-emptying stimulus. After the recording of an initial baseline in 2 mM Ca\textsuperscript{2+}, and then for 5 min in nominally Ca\textsuperscript{2+}-free buffer, the addition of BK (1 \(\mu\)M) caused a near instantaneous increase in \([\text{Ca}^{2+}]_{i}\). This decayed back to the baseline within \(-2\) min; a response

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**Bronchoconstrictors enhance SrcFK and FAK auto-phosphorylation.** In order to confirm that the influence of SrcFK and FAK on contraction and Rho-kinase activity occurs in direct response to bronchoconstrictor stimulation, we also examined the effects of BK or CCh on SrcFK auto-phosphorylation at Y416 and FAK auto-phosphorylation at Y397, as a reflection of respective changes in kinase activity (Calalb et al., 1995; Xu et al., 1999). In hASMC, auto-phosphorylation of both kinases was significantly enhanced by both agents (Figure 3 A–D). As expected, SrcFK phosphorylation was almost abolished by PP2, and FAK phosphorylation was almost abolished by PF-573228, both confirming the selectivity of the phospho-antibodies and

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Y Shaifta et al.
Figure 2
Effects of kinase inhibitors on contraction in α-toxin permeabilized rat bronchioles and MLC20/MYPT-1 phosphorylation in hASM. (A–C) Measurement of isometric tension in α-toxin permeabilized rat bronchioles. All responses were performed with pCa fixed at 6.5 and in the presence of 10 μM CPA and 1 μM GTP. (A) Representative traces showing CCh (upper panel) or BK (lower panel) being applied cumulatively (0.01–100 μM) at 5 min intervals, with arrows indicating where the first dose was applied. Responses were performed in the absence of inhibitor (control) or after pre-incubation with either the Src inhibitor PP2 (30 μM, 10 min), the Rho-kinase inhibitor Y27632 (10 μM, 10 min) or the FAK inhibitor PF-573228 (10 μM, 10 min). Measurements were taken at the end of each 5 min exposure. Data are expressed as a % of that induced by pCa 4.5 (mean ± SEM). (B) CCh data were fitted by nonlinear regression (control, n = 10; PP2, n = 9; Y27632, n = 4; PF-573228, n = 11); see main text for effects on CCh PD2. (C) BK data could not be fitted by nonlinear regression so were compared by two-way RM ANOVA (control, n = 6; PP2, n = 6; Y27632, n = 4; PF-573228, n = 6; *P < 0.05, **P < 0.01 vs. control). (D–G) Measurement of phosphorylation of MLC20 at S19 (P-MLC20, D, E) and MYPT-1 at T696 (P-MYPT-1, F, G) in hASM. Representative blots show effects of treatment on ‘phospho’ and ‘total’ immunoreactivity for each protein. Bar charts show the effects of treatments on the degree of phosphorylation (mean ± SEM), expressed as a % of values from untreated (control) samples run on the same gels. (D) Effects of BK (1 μM 30 s) on MLC20 phosphorylation in the absence of inhibitor (n = 16) or after pretreatment with either PP2 (30 μM, 10 min, n = 11), Y27632 (10 μM, 10 min, n = 9) or PF-573228 (PF, 10 μM, 10 min, n = 11). (E) Effects of inhibitors on basal MLC20 phosphorylation (n = 4–11). (F) Effects of BK (1 μM, 30 s) on MYPT-1 phosphorylation in the absence of inhibitor (n = 13), or after pre-application of PP2 (30 μM, 10 min, n = 13), Y27632 (10 μM, 10 min, n = 13), or PF-573228 (10 μM, 10 min, n = 8). (G) Effects of inhibitors on basal MYPT-1 phosphorylation (n = 4–11). Comparisons were by one-way ANOVA with Holm–Sidak post tests: *P < 0.05 and **P < 0.01 versus control; #P < 0.5 and ###P < 0.01 versus BK alone.
2 mM Ca\(^{2+}\) was then re-applied for 20 min, with simultaneous washout of BK. This induced a biphasic rise in [Ca\(^{2+}\)]\(_i\) with a similar time course to the SOCE contractile responses. In the presence of either PP2 or PF-573228, the sustained component was reduced by ~50%, while the initial transient component and the initial BK-induced SR release were both unaffected (Figure 5).

**Figure 3**
Effects of bronchoconstrictors on SrcFK and FAK auto-phosphorylation in hASMC and rat trachealis. (A–D) Measurements of auto-phosphorylation of SrcFK at Y416 (P-SrcFK, A, B) and auto-phosphorylation of FAK at Y397 (P-FAK, C, D) in hASMC. Representative blots show effects of treatments on ‘phospho’ and ‘total’ immunoreactivity for each protein. Bar charts show the effects of treatments on the degree of phosphorylation (mean ± SEM), expressed as a % of values from untreated (control) samples run on the same gels. (A) Effect of BK (1 μM, 30 s) in the absence (n = 12) or presence of PP2 (30 μM, 10 min, n = 7) on P-SrcFK (Y416). (B) Effect of CCh (100 μM, 30 s) in the absence (n = 13) or presence of PP2 (n = 8) on P-SrcFK (Y416). (C) Effect of BK in the absence (n = 15) or presence of PF-573228 (PF, 10 μM, 10 min, n = 13) on P-FAK (Y397). (D) Effect of CCh in the absence (n = 16) or presence of PF-573228 (n = 11) on P-FAK (Y397). Comparisons by one-way ANOVA with Holm–Sidak post tests: **P < 0.01 versus control; ##P < 0.01 versus BK or CCh alone. (E, F) Measurements of phosphorylation of MLC\(_{20}\) (S19), MYPT-1 (T693), SrcFK (Y416) and FAK (Y397) in rat trachealis muscle. (E) Effects of BK (1 μM, 30 s, n = 8). (F) Effects of CCh (100 μM, 30 s, n = 8). Comparisons by unpaired t-test: *P < 0.05, **P < 0.01 versus control.
light of previous evidence suggesting cooperation between the two kinases, we investigated the influence of FAK on SrcFK auto-phosphorylation and vice versa. Enhancement of SrcFK (Y416) auto-phosphorylation by BK was inhibited by PF-573228 by ~50% (Figure 6A), while basally, this phosphorylation was insensitive to PF-573228 but inhibited by PP2 (Figure 6B). FAK kinase activity is also reportedly influenced by Src-dependent phosphorylation on FAK Y576/577 (Calalb et al., 1995). Phosphorylation at this dual site was enhanced by BK, and this enhancement was nearly abolished by PP2 and partially inhibited by PF-573228 (Figure 6C), while basally, this phosphorylation was inhibited by PP2, but not PF-573228 (Figure 6D). However, BK-induced enhancement of FAK (Y397) auto-phosphorylation was

Figure 4
Effects of SrcFK and FAK inhibitors on VOCE- or SOCE-associated contraction in rat bronchioles. (A, B) VOCE-associated contractions induced by 40 mM KPSS. Representative traces (A) and mean measurements of peak amplitude (B, ± SEM), showing effects of two contractions in the absence of inhibitor (left panels, n = 8), the effect of PF-573228 on the second contraction (PF, middle panels, 10 μM, 5 min pre-incubation, n = 7) or the effect of PP2 on the second contraction (30 μM, right panels, 5 min pre-incubation, n = 9). Comparisons by paired t-test: **P < 0.01 versus control. (C, D) SOCE-associated contraction induced by 10 μM CPA/200 μM EGTA/zero Ca²⁺, followed by re-application of 2 mM Ca²⁺. Representative traces (C) and mean measurements of amplitude of contractile responses at 2 min intervals after re-application of Ca²⁺ (D, ± SEM), showing control response (n = 14) and effects of pre-incubation with PF-573228 (n = 9), PP2 (n = 9), nifedipine (n = 10) or YM58483 (n = 5). Comparisons by two-way ANOVA with Holm–Sidak post tests: *P < 0.05 for control versus PF-573228 or PP2. #P < 0.01 for control versus YM58483. ^P < 0.05 for control versus nifedipine at 2 min only.
unaffected by PP2 (Figure 6E). Basal phosphorylation of FAK (Y397) was also insensitive to PP2, but was inhibited by PF-53228 (Figure 6F).

c-Src is the principle SrcFK mediating bronchoconstrictor-induced phosphorylation responses. Multiple members of the Src-family of kinases are expressed in ASM, including c-Src, Fyn, Yes and Lyn (Sakai et al., 2010). For this reason, and the fact that the phospho-SrcFK antibody cannot distinguish between Src family members, we re-examined the effects of acute BK treatment on MLC20 (S19), MYPT-1 (T696), SrcFK (Y416) and FAK (Y576/577) phosphorylation in hASM after transfection with c-Src siRNA or scrambled siRNA. Specificity of c-Src siRNA was verified by a ~70% reduction in c-Src FK phosphorylation in siRNA-treated cells compared to scrambled siRNA controls.

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**Figure 5**

Effects of SrcFK and FAK inhibitors on SOCE/ROCE-associated \([Ca^{2+}]_o\) responses in hASM. A: Representative control trace of \([Ca^{2+}]_o\) in Fura-2 loaded hASM, as determined by the ratio of fluorescence at 340 nm/380 nm. Arrow indicates the point at which pre-stimulus basal \([Ca^{2+}]_o\) was recorded in 2 mM \([Ca^{2+}]_o\), (and extrapolated by dashed line). The buffer was then switched to zero \([Ca^{2+}]_o\) until a new baseline was established, and 1 μM BK added for 5 min. Finally, 2 mM \([Ca^{2+}]_o\), was re-applied for 20 min. Responses were performed either in the absence or presence of FAK inhibitor PF-573228 (B, C: PF, 10 μM, added 5 min prior to BK, n = 15 vs. 15 matched controls) or SrcFK inhibitor PP2 (D, E: 30 μM, added 5 min prior to BK, n = 10 vs. eight matched controls). Measurements were made of the peak BK-induced transient (B, D: arbitrary units, mean ± SEM) and of the response to reapplication of 2 mM \([Ca^{2+}]_o\) (C, E: arbitrary units, measured at 1 min intervals, mean ± SEM), compared with the pre-stimulus basal \([Ca^{2+}]_o\), in 2 mM \([Ca^{2+}]_o\), (indicated by arrows). Background fluorescence (in zero \([Ca^{2+}]_o\), prior to the application of BK) was subtracted from all other measurements. Comparisons by un-paired t-test (B, D) or two-way ANOVA with Holm–Sidak post tests (C, E: *P < 0.05 vs. matched controls).
protein expression, while expressions of MLC20, MYPT-1 and FAK were all unaffected (Figure 7A). The scrambled siRNA had no effect on any of the proteins examined. c-Src siRNA inhibited BK-induced phosphorylation of MLC20 by ~80%, while responses of MYPT-1 (T696), SrcFK (Y416) and FAK (Y576/577) to BK were all inhibited by ~60%, compared with matched scrambled siRNA transfected cells (Figure 7 B–D).

Discussion

We examined the role of SrcFK and FAK kinase activity in bronchoconstrictor-induced contraction of rat-isolated bronchioles and in [Ca^{2+}]_i and phosphorylation responses in hASMC. Contraction was induced and MLC20 phosphorylation was enhanced by the bronchoconstrictors BK and
CCh, and these responses were sensitive to inhibition of both SrcFK and FAK. Using auto-phosphorylation as an indication of kinase activity, both SrcFK and FAK were activated by both agents in hASMC and rat trachealis, suggesting an important role for these kinases in GPCR-induced ASMC contraction in both humans and rodents. PP3, the negative control for PP2, was without effect on contraction, while PF-431396, another inhibitor of FAK, had similar effects as PF-573228, reducing the likelihood of our results being influenced by non-specific effects of PP2 or PF-573228. Amongst the several Src family members, both c-Src and Fyn have been implicated in vascular smooth muscle contraction (Nakao et al., 2002; Knock et al., 2008), and Lyn is activated by muscarinic agonists in ASM (Pertel et al., 2006). Here however, the effects of c-Src siRNA suggest that 60–80% of all the bronchoconstrictor-induced phosphorylation responses investigated herein were specifically mediated by c-Src.

Figure 7
Effects of c-Src siRNA on bronchoconstrictor-induced MLC20, MYPT-1, SrcFK and FAK phosphorylation in hASMCs. Measurement of protein expression and phosphorylation responses to BK (1 μM, 30 s) in hASMCs after transfection with c-Src siRNA or scrambled siRNA (scram). (A) Effect of c-Src siRNA on c-Src protein expression and (lack of) effect on MLC20, MYPT-1 and FAK expression. Data normalized to GAPDH expression in the same samples (arbitrary units, n = 8–12). Comparisons by unpaired t-test: **P < 0.01 versus scram siRNA. (B) Effect of BK on MLC20 phosphorylation at S19 (P-MLC20) after transfection with scram siRNA (n = 16) or c-Src siRNA (n = 16). (C) Effect of BK on MYPT-1 phosphorylation at T696 (P-MYPT-1) after transfection with scram siRNA (n = 14) or c-Src siRNA (n = 14). (D) Effect of BK on SrcFK phosphorylation at Y416 (P-SrcFK) after transfection with scram siRNA (n = 16) or c-Src siRNA (n = 16). (E) Effect of BK on FAK phosphorylation at Y576/577 (P-FAK) after transfection with scram siRNA (n = 10) or c-Src siRNA (n = 10). Comparisons by two-way ANOVA: **P < 0.01 versus control; ###P < 0.01 versus scram siRNA.
Tyrosine kinases in airway smooth muscle

To further characterize the signalling pathways through which SrcFK and FAK mediate ASM contraction, we first focussed on their role in Rho-kinase dependent Ca\(^{2+}\)-sensitization, a process whereby inhibition of MLCP results in depolarization-induced opening of L-type Ca\(^{2+}\) channels STIM1/Orai1/TRP-dependent in (VOCE) (Kawasaki et al., 2003). Both BK and CCh-induced contraction were highly sensitive to Rho-kinase inhibition with Y27632. Furthermore, a component of the contractile response to both BK and CCh persisted when bronchioles were permeabilized with \(\alpha\)-toxin to prevent changes in intracellular Ca\(^{2+}\). We found that these contractile responses were dependent on Rho-kinase and SrcFK, but not FAK. Furthermore, we found that MYPT1 phosphorylation on T696, an indicator of Rho-kinase-mediated MLCP inhibition (Feng et al., 1999), is also enhanced by BK and CCh and that this enhancement is sensitive to inhibition of Rho-kinase and SrcFK, but not of FAK. This influence of SrcFK on Rho-kinase activity occurred specifically in response to agonist stimulation, because baseline phosphorylation of MYPT1 and MLC\(_{20}\) and baseline pCa6.5 contraction in permeabilized bronchioles were not affected by SrcFK inhibition. Clearly, these results indicate that SrcFK mediates GPCR-induced smooth muscle contraction in part via activation of Rho-kinase. Importantly, this is the first direct demonstration of an interaction between SrcFK and Rho-kinase in ASM and in vessels of a size relevant to the control of airway resistance, consistent with the implied importance of Rho-kinase in airway hyper-responsiveness from studies in whole animal or isolated upper airways (Yoshii et al., 1999; Schaafsma et al., 2006). Interestingly, receptor TK stimulation also induces Rho-kinase-dependent ASM contraction (Gosens et al., 2004), and other responses of ASM to growth factor stimulation are also SrcFK-dependent (Krymskaya et al., 2005). Thus SrcFK may be a point of convergence for the activation of Rho-kinase in response to either GPCR or growth factors.

Rho-kinase is directly activated by the small G-protein RhoA, which is itself activated by guanine nucleotide exchange factors (RhoGEFs). RhoGEFs are known to be activated or modulated by various non-receptor TKs (Chikumi et al., 2002; Ying et al., 2009; Guillevill et al., 2010), in addition to G\(_{12/13}\) binding. It is therefore conceivable that SrcFK may be activating RhoA/Rho-kinase via direct phosphorylation of a RhoGEF. Alternatively, they may do so via the prior activation of another kinase, such as FAK, PYK2 or JAK2 (Calalb et al., 1995; Andreev et al., 2001; Singh et al., 2011). Our results are not inconsistent with this hypothesis, but exclude FAK as the intermediary kinase in this instance.

We next focussed on the role of SrcFK and FAK in Ca\(^{2+}\) signalling. Gq/PLC-\(\beta\)-coupled GPCRs induce Ca\(^{2+}\) entry through three main pathways: DAG-sensitive TRP channel opening (ROCE), IP\(_{3}\)-dependent depletion of SR Ca\(^{2+}\) and subsequent STIM1/Orai1/TRP-dependent influx (SOCE), and subsequent depolarization-induced opening of L-type Ca\(^{2+}\) channels (VOCE) (Kawasaki et al., 2006; Wang et al., 2008). Several members of the TRPC family of channels, in addition to being modulated by DAG, are subject to modulation by phosphorylation, and tyrosine phosphorylation of TRPC channels is SrcFK-dependent, contributing to either ROCE or SOCE (Kawasaki et al., 2006). Moreover, association of STIM1 with Orai1 in response to SR depletion, and subsequent Ca\(^{2+}\) influx, is partially dependent on SrcFK-mediated phosphorylation of STIM1 (Lopez et al., 2012). In accord with these previous studies, we found that CPA-induced SOCE-dependent contraction in rat bronchioles and BK-induced Ca\(^{2+}\) influx in hASMC were both similarly sensitive to SrcFK inhibition. Interestingly, we found that these responses were also similarly sensitive to FAK inhibition. This, to our knowledge, is the first indication that FAK may be contributing to GPCR-induced Ca\(^{2+}\) responses and contraction in human and rodent ASM, via upstream modulation of SOCE and/or ROCE. Some GPCR agonists, notably angiotensin II, also mediate SR Ca\(^{2+}\) release via SrcFK-dependent tyrosine phosphorylation of PLC-\(\gamma\) (Schmitz et al., 1997). However, there is no indication that this is occurring in our study, because we found that neither PP2 nor PF-573228 altered the BK-induced Ca\(^{2+}\) transients indicative of SR release.

Our finding that the CPA-induced SOCE contraction was minimally affected by the Ca\(^{2+}\) channel antagonist nifedipine suggests that VOCE secondary to SOCE-induced depolarization was not contributing substantially to this response. However, VOCE may also be activated more directly via a number of signalling pathways including stretch-activated phosphatidylcholine-specific PLC-derived DAG (Mauban et al., 2015) or integrin-directed tyrosine phosphorylation. Regarding the latter, engagement of integrin \(\alpha\)5\(\beta\)1 induces SrcFK and FAK-dependent phosphorylation of the a1c subunit of the L-type Ca\(^{2+}\) channel in vascular smooth muscle, with the likely sequence of events being integrin-induced FAK auto-phosphorylation followed by SrcFK recruitment by FAK (Owen et al., 1999; Salazar and Rozengurt, 2001) and subsequent direct phosphorylation of the channel by SrcFK (Wu et al., 2001; Gui et al., 2006). Similarly in ASM, VOCE may also be enhanced via stretch or adhesion-induced FAK activity (Smith et al., 1998; Tang et al., 1999). In bronchioles, we found that contraction induced by depolarization with sub-maximal (40 mM) K\(^{+}\) was sensitive to PF-573228 but not PP2, which suggests that FAK may be activating VOCE independently of SrcFK. However, contraction induced by maximum depolarization with 80 mM K\(^{+}\) was considerably less sensitive to PF-573228, ruling out a non-specific effect of PF-573228 on Ca\(^{2+}\) channel opening per se. We did not systematically examine the effects of stretch on contractile responses, as carried out previously in trachea (Tang et al., 1999), but applied a standard degree of stretch to maximize active tension responses to bronchoconstrictors or KPSS. Similarly, adherent hASMC are assumed to be under a degree of self-induced basal tension (Deguchi et al., 2005). In light of this, it is worth noting that the relatively weak FAK auto-phosphorylation observed in trachealis samples treated with BK or CCh, compared with similarly treated hASMC, may have been because no stretch was applied to trachealis or isolated upper airways. In accord with these previous studies, we found that CP A-induced SOCE-dependent contraction in rat bronchioles and BK-induced Ca\(^{2+}\) influx in hASMC were both similarly sensitive to SrcFK inhibition. Interestingly, we found that these responses were also similarly sensitive to FAK inhibition. This, to our knowledge, is the first indication that FAK may be contributing to GPCR-induced Ca\(^{2+}\) responses and contraction in human and rodent ASM, via upstream modulation of SOCE and/or ROCE. Some GPCR agonists, notably angiotensin II, also mediate SR Ca\(^{2+}\) release via SrcFK-dependent tyrosine phosphorylation of PLC-\(\gamma\) (Schmitz et al., 1997). However, there is no indication that this is occurring in our study, because we found that neither PP2 nor PF-573228 altered the BK-induced Ca\(^{2+}\) transients indicative of SR release.

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In smooth muscle contraction, it has been assumed that the mutual dependence between SrcFK and FAK relates primarily to the recruitment of contractile fibres through actin polymerization and focal attachment formation (Gerthoffer and Gunst, 2001; Tang et al., 1999; Gunst et al., 2003). It is therefore of note that we only see possible evidence of such mutuality with regard to SOCE/ROCE activity, but not in
In conclusion, our data suggest an important role for SrcFK and FAK in bronchoconstrictor-mediated contraction in ASM, with the two kinases acting together to induce SOCE/ROCE, and independently to mediate Rho-kinase-dependent Ca²⁺ sensitization and VOCE respectively. These findings may inform the search for new drug targets for the treatment of obstructive lung diseases such as asthma, and in particular, support the suggested key role for SrcFK in experimental airway hyper-responsiveness (Sakai et al., 2010; Katsumoto et al., 2013).

Acknowledgements

Thanks to Mrs Kheem Jones and other NHS staff for patient recruitment and provision of bronchoscopy biopsies. This work was supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. The views expressed are those of the author(s) and not necessarily those of the NHS, NIHR or the Department of Health.
Tyrosine kinases in airway smooth muscle


Owen JD, Ruest PJ, Fry DW, Hanks SK (1999). Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and

Funding

Wellcome Trust: #087776. British Heart Foundation: FS/12/43/29608.

Author contributions

All other authors reviewed the manuscript critically for important intellectual content. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of interest

Authors declare that they have not any conflict of interest.

References


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

http://dx.doi.org/10.1111/bph.13313

**Figure S1** Identification of hASMC as smooth muscle by positive staining with anti-smooth muscle-actin (panel A), anti-desmin (panel B) and anti-calponin (panel C), visualised with Alexa Fluor®488 labelled secondary antibody (Lifetechnologies) and fluorescent microscopy. Cells were also stained with TRITC-labelled phalloidin to confirm the presence of stress fibres in resting cells (Panel D). In Panel D, nuclei are visualised by staining with Hoechst. Scale bar =20μm.

**Figure S2** Effect of PP3 on contractile responses in rat bronchioles.

**Figure S3** Effect of PF-431396 on contractile responses in rat bronchioles.

**Figure S4** Effects of SrcFK, Rho-kinase and FAK inhibition on bradykinin-induced contractile responses in rat bronchioles.